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***Orientia tsutsugamushi*, the causative agent of scrub typhus,
induces an inflammatory program in human macrophages**

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Abbreviations: fetal bovine serum, FBS; Fold Change, FC; Gene Ontology, GO; heat-killed,
HK; interferon, IFN; interferon-stimulated gene, ISG; oligomerization domain, NOD;
quantitative real-time PCR, qPCR; TdT-mediated dUTP nick-end labeling, TUNEL; Tumor
Necrosis Factor, TNF.

Abstract

Scrub typhus is a life-threatening disease caused by *Orientia tsutsugamushi*, a bacterium that primarily infects endothelial cells both *in vitro* and *in vivo*. Evidence suggests that the interaction of *O. tsutsugamushi* with myeloid cells may play a pivotal role in *O. tsutsugamushi* infection. We demonstrated that *O. tsutsugamushi* replicated within human monocyte-derived macrophages. Bacteria stimulated the expression of a large number of genes, including type I interferon genes, interferon-stimulated genes, inflammation-associated genes and apoptosis-related genes, and the release of inflammatory cytokines such as Tumor Necrosis Factor and interleukin-1 β . In addition, *O. tsutsugamushi* induced an M1-type genetic program in macrophages. *O. tsutsugamushi* viability was required for the type I interferon response and, to a lesser degree, for the inflammatory response. As interferon- γ is known to elicit M1 polarization, we assessed the effect of interferon- γ on the fate of *O. tsutsugamushi* in macrophages. Exogenous interferon- γ partially inhibited *O. tsutsugamushi* replication within macrophages. Our results suggest that the inflammatory response induced by *O. tsutsugamushi* may account for the local and systemic inflammation observed in scrub typhus.

Keywords: *Orientia tsutsugamushi*; macrophages; type I interferon; inflammatory cytokines; chemokines; apoptosis.

1. Introduction

Orientia tsutsugamushi is the causative agent of scrub typhus, a tropical infectious disease endemic to the Asia-Pacific region, and is responsible for one million new infections each year. *O. tsutsugamushi* is transmitted to humans by the bite of larval trombiculid mites, and an estimated one billion people are at risk of contracting the infection [1]. Scrub typhus is associated with fever, rash and eschar. The clinical forms of scrub typhus include pneumonitis and meningitis, which may lead to multiorgan failure and death [2]. To date, no effective preventive strategy can provide long-lasting immunity, despite attempts to develop a prophylactic vaccine for scrub typhus [3].

O. tsutsugamushi belongs to a genus that has been separated from the genus *Rickettsia* on the basis of genetic and phenotypic differences, which include the absence of peptidoglycan and lipopolysaccharide in its cell wall [4]. The genomes of the Boryong and Ikeda strains have recently been sequenced [5, 6]. *O. tsutsugamushi* is considered to be an intracellular pathogen that primarily infects endothelial cells both *in vitro* [7] and *in vivo* [8]. In addition, it appears that the interaction of *O. tsutsugamushi* with myeloid cells plays a pivotal role in *O. tsutsugamushi* infection [9]. In mice, *O. tsutsugamushi* infects and multiplies in peritoneal macrophages [10, 11], leading to the death of infected animals. However, mice are able to resist to infection if pretreated with a macrophage-activating agents such as BCG [12]. In humans, tissues autopsied from patients showing clinical symptoms of scrub typhus revealed *O. tsutsugamushi* in macrophages from the liver and the spleen [8]. We showed that *O. tsutsugamushi* replicates in human monocytes isolated from healthy donors and that infection alters the expression of a large number of host genes [13]. These findings were strengthened by recently published data demonstrating the tropism of *O. tsutsugamushi* for monocytes and dendritic cells within eschars [14].

1 The mechanisms that govern local and systemic inflammation in scrub typhus have
2 only been partially elucidated. In patients with scrub typhus, the circulating levels of
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4 inflammatory cytokines and chemokines are elevated [15-17]. Similarly, the susceptibility of
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6 mice to *O. tsutsugamushi* infection is associated with the overproduction of cytokines and
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8 chemokines [9, 18]. The expression of genes associated with the inflammatory response and
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10 of the interferon-stimulated genes (ISGs) is upregulated in human monocytes stimulated with
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12 *O. tsutsugamushi*. In addition, several features of M1 polarization are observed in circulating
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14 leukocytes from patients with scrub typhus [13]. As the half-life of monocytes in the blood is
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16 limited, the infection of tissue macrophages by *O. tsutsugamushi* may be independent of
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18 circulating cells.
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24 In this study, we demonstrate that *O. tsutsugamushi* is able to replicate within human
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26 monocyte-derived macrophages and that it induces a robust transcriptional response in ISGs,
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28 apoptosis-related genes and genes encoding inflammatory mediators. Infection with *O.*
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30 *tsutsugamushi* also induces the release of interferon (IFN)- β and inflammatory cytokines.
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32 Although this exacerbated inflammatory response is not sufficient to eradicate *O.*
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34 *tsutsugamushi*, it may account for the inflammatory complications of scrub typhus.
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41 **2. Materials and Methods**

43 *2.1. Bacteria and cell culture*

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45 *O. tsutsugamushi*, strain Kato (CSUR R163), was propagated and titered in L929 cells as
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47 described [13]. In brief, infected cells were lysed using glass beads and centrifuged to remove
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49 cell debris, and bacteria were frozen in medium containing 20% fetal bovine serum (FBS,
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51 Invitrogen, Cergy Pontoise, France) and 5% dimethylsulfoxide until use. The bacterial titer
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53 was determined by serial dilutions of bacterial preparations, and quantified using indirect
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immunofluorescence (see below). In some experiments, *O. tsutsugamushi* were killed by heating at 100°C for 5 minutes (HK bacteria).

Peripheral blood mononuclear cells were isolated from leukopacks (Etablissement Français du Sang, Marseille, France) over a Ficoll gradient (MSL, Eurobio, Les Ulis, France). Monocytes were isolated by adherence in 24-well plates with or without 12-mm glass coverslips and were differentiated into macrophages using a previously described procedure [19] with slight modifications. Briefly, monocytes were cultivated in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine (Invitrogen) and 10% heat-inactivated human AB serum (Lonza, Verviers, Belgium) for three days, and human serum was replaced by 10% FBS for additional periods ranging from an additional 4 to 6 days. Cultivated cells were designated as monocyte-derived macrophages as more than 90% of the cells did not express CD14 but did express CD68 as assessed by flow cytometry (Fig. S1). In some experiments, macrophages were treated with 50 ng/ml of recombinant human IFN- γ (Peprotech France, Neuilly-sur-Seine) for 24 hours before infection, and IFN- γ was added to cell preparations every day.

2.2. Infection procedure

Macrophages (1.5×10^5 cells per assay) were incubated in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine and 10% heat-inactivated FBS in the presence of *O. tsutsugamushi* at a bacterium-to-cell ratio of 10:1 for 2 hours, a dose sufficient to determine bacterial uptake by monocytes [13]. Macrophages were washed to eliminate unbound bacteria and were cultivated for different time periods. The uptake and the intracellular fate of the bacterial pathogens was determined using confocal microscopy and quantitative real-time PCR (qPCR), as recently described [13]. For confocal microscopy, macrophages were infected for two hours, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated with Alexa Fluor 488-phalloidin (Invitrogen) to stain filamentous actin and

1 pooled serum from Thai patients with scrub typhus (1:400 dilution). Secondary antibodies
2 consisted of goat anti-human IgG conjugated with Alexa Fluor 555 (Invitrogen).
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4 Macrophages then mounted with Mowiol and were examined using a Leica TCS SP5
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6 confocal microscope with a 63X/1.32-0.6 oil CS lens and an electronic 1.5X zoom. Images
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8 were processed with Adobe Photoshop V5.5 software. To isolate bacterial DNA,
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macrophages were lysed using 0.1% Triton X-100, and DNA was extracted in a 100 µl
volume using a QIAamp Tissue Kit (Qiagen, Courtaboeuf, France) and was stored at -20°C.
qPCR was performed using 5 µl of DNA extract, specific primers and probes and the Taqman
system (Applied Biosystems, Warrington, UK). Primers and probe were constructed
according to the available DNA sequence of *O. tsutsugamushi* strain Boryong (complete
genome, GenBank ref. NC_009488.1). The forward primer consisted of 5'-
AAGCATAGGTTACAGCCTGGWGA-3'; the reverse primer consisted of 5'-
ACCCCAACGGATTTAATACTATATCWAC-3'; the probe consisted of 5'-FAM-
CCATCTTCAAGAAATGGCATATCTTCCTCAGG- TAMRA-3'. Each qPCR run included
a standard curve generated from tenfold serial dilutions of a known concentration of bacterial
DNA (Fig. S2), and a negative control consisted of DNA extracted from uninfected
macrophages. The results are expressed as the total bacterial DNA copy number.

2.3. Microarrays

Macrophages (4×10^5 cells per assay) were incubated with *O. tsutsugamushi* at a bacterium-
to-cell ratio of 10:1 for 8 hours. RNA was extracted from infected and uninfected
macrophages using an RNeasy Mini kit (Qiagen) with a DNase digestion step. The quality of
isolated RNAs was assessed using Agilent 2100 Bioanalyzer and an RNA 6000 Nano Kit
(Agilent Technologies, Massy, France), and the quantity of RNAs was assessed using a
NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Eight RNA

1 samples (four samples per experimental condition) were reverse transcribed in cDNA, and
2 cDNA was labeled with Cy3 using a Low RNA Input Fluorescent Linear Amplification Kit
3 (Agilent Technologies, Massy, France). The cDNA was hybridized to 4×44K microarray
4 slides (Agilent Technologies). Scanned images were analyzed using Feature Extraction
5 Software 10.5.1 (Agilent Technologies), and data processing and analyses were performed
6 using Resolver 7.1 software (Rosetta Inpharmatics, Cambridge, MA), R software and
7 BioConductor libraries, as described [13]. The Rosetta intensity error model for single color
8 microarrays was used to normalize inter-array values. Statistical supervised analysis was
9 performed using the significance analysis of microarrays, and the genes displaying an
10 absolute fold change (FC) greater than 2.0 and a False Discovery Rate value less than 0.01
11 were considered to be differentially expressed. Results are expressed as FC calculated from
12 the average of the four samples of unstimulated and *O. tsutsugamushi*-stimulated
13 macrophages. The differentially expressed genes were classified on the basis of their Gene
14 Ontology (GO) category as assessed using the DAVID bioinformatics tools [20]. To assess
15 the M1/M2 polarization status of macrophages stimulated with *O. tsutsugamushi*, 30 genes
16 known to be associated with M1 polarization and 22 M2 genes were manually selected from
17 the literature [21-23]. Data were entered in the ArrayExpress database according to the
18 Minimum Information About a Microarray Experiment procedure and can be accessed using
19 the Gene Expression Omnibus series accession number at
20 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38194>.
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51 *2.4. Quantitative real time RT-PCR*

52 Quantitative real time RT-PCR (qRT-PCR) was performed as described previously [13]. In
53 brief, total RNA was isolated from macrophages using a Qiagen kit, and cDNA was
54 synthesized using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen). PCR
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was carried out using specific primers designed with primer3 software [24] (primers are the list in Table 1) and with Light Cycler-FastStart DNA MasterPLUS SYBR Green I master mix (Roche Applied Science, Meylan, France). We verified that the expression of the housekeeping gene encoding β -actin was not modulated in macrophages stimulated with *O. tsutsugamushi*, and we calculated the FC values in the expression of genes relative to the expression of β -actin gene using the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = (C_t\text{Target} - C_t\text{Actin})_{\text{stimulated macrophages}} - (C_t\text{Target} - C_t\text{Actin})_{\text{unstimulated macrophages}}$ and C_t values were defined as the number of cycles for which the fluorescence signals were detected [19].

2.5. *TdT-mediated dUTP nick-end labeling (TUNEL) assay*

Infected macrophages were fixed with 3% paraformaldehyde, and cell death was determined using In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) as described previously [13]. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used to counter-stain nuclei. The number of TUNEL-positive cells and the number of DAPI-stained nuclei were determined using fluorescence microscopy. Cell death is expressed as the number of TUNEL-positive cells relative to the number of DAPI-stained nuclei.

2.6. *Immunoassays*

The levels of Tumor Necrosis Factor (TNF) and IFN- β in macrophage supernatants were determined using TNF and IFN- β ELISA kits, respectively (R&D Systems, Lille, France). IL-1 β levels were measured using a specific ELISA kit provided by Diaclone (Besançon, France). The detection limit of the kits was 5 pg/ml for TNF, 10 pg/ml for IL-1 β , and 8 pg/ml for IFN- β . The intra- and interassay coefficients of variation of the ELISA kits ranged between 5 and 10%.

2.7. Statistical analysis

Results were compared using the non-parametric Mann-Whitney U test and are expressed as means \pm standard deviation (SD). Differences were considered significant when p values were less than 0.05.

3. Results

3.1. Intracellular fate of *O. tsutsugamushi* in macrophages

The uptake of *O. tsutsugamushi* by macrophages was determined by confocal microscopy. Bacteria could be detected within macrophages after 30 minutes, and several bacteria were present in macrophages after two hours (Fig. 1A). The intracellular replication of *O. tsutsugamushi* was determined by incubating macrophages with organisms (10 bacteria per macrophage) for two hours and removing free bacteria by washing (considered to be day 0). Infected macrophages were cultured for an additional 5 days. At day 0, the number of bacterial DNA copies was $6.9 \pm 1.5 \times 10^4$ and increased approximately twofold each subsequent day to reach $35 \pm 2 \times 10^5$ bacterial DNA copies after 5 days (Fig. 1B). Therefore, *O. tsutsugamushi* is able to replicate in macrophages.

3.2. Global transcriptome analysis of *O. tsutsugamushi*-infected macrophages

To understand how *O. tsutsugamushi* replicates within macrophages, we compared the transcriptional profile of macrophages stimulated with *O. tsutsugamushi* for 8 hours to that of unstimulated macrophages using whole genome microarrays. We found that 379 probes (364 upregulated and 15 downregulated probes) were modulated in response to *O. tsutsugamushi* infection. The hierarchical clustering computed from modulated genes revealed that *O. tsutsugamushi*-stimulated macrophages were markedly different from control macrophages (Fig. 1C). The annotation of the modulated genes was enriched for functions related to the

immune response and host defense (e.g. immune response, chemotaxis, regulation of cytokine production, leukocyte activation, regulation of lymphocyte activation, regulation of immune effector process, cytokines and inflammatory response), apoptosis and cell signaling (TLR signaling pathway) (Fig. 1D). These data demonstrate that *O. tsutsugamushi* stimulates the expression of specific genes that generate a transcriptional signature, including genes involved in the host response to infection.

3.3. Analysis of the responses induced by *O. tsutsugamushi*

From the macrophage genes that were differentially expressed in response to *O. tsutsugamushi*, we selected genes for further characterization belonging to the categories described above and that have been reported to be modulated in human monocytes stimulated with *O. tsutsugamushi* [13]. These genes included those associated with the type I IFN pathway, the inflammatory response and apoptosis.

The differential modulation of genes encoding IFN- β and ISGs, such as OAS1 and MX1, which were upregulated in the microarray, was confirmed by qRT-PCR using the macrophages from three different donors stimulated with *O. tsutsugamushi*. The upregulated expression of these genes was long lasting, as the increase remained evident after 18 hours (Fig. 2A). This type I IFN response of *O. tsutsugamushi*-stimulated macrophages was also observed at the protein level. While unstimulated macrophages were unable to release IFN- β (less than 8 pg/ml), the amount of IFN- β released by macrophages in response to *O.*

tsutsugamushi was of 33 ± 9 and 40 ± 3 pg/ml after 24 and 48 hours, respectively (Fig. 2E).

We likewise selected genes related to inflammation that were upregulated in the microarray and analyzed them by qRT-PCR. We found that genes associated with chemotaxis (CCL5, CCL20, CXCL10 and CXCL11) were upregulated after 8 and 18 hours of stimulation with *O. tsutsugamushi* (Fig. 2B). Genes encoding inflammatory cytokines (TNF, IL-1 β , IL-16, IL-

12p40, IL-23p19 and IL-15) were also upregulated; however this upregulation was transient for the gene encoding IL-1 β (Fig. 2C). It is possible that this robust modulation of inflammatory genes is associated with the M1 polarization of macrophages. To address this, we compared the transcriptional profile of *O. tsutsugamushi*-infected macrophages with 30 genes representative of M1 macrophages and 22 genes representative of M2 macrophages [21-23]. Of the M1-associated genes, 18 were upregulated (Table 2), while only 2 of the M2-associated genes were upregulated following *O. tsutsugamushi* infection (Table 3). The majority of M2-associated genes were downregulated (Table 3), demonstrating that *O. tsutsugamushi* induces an M1-like transcriptional profile in macrophages. Similar to the type I IFN data, this change in the expression of inflammatory genes was reflected at the protein level. Unstimulated macrophages released about 40 pg/ml TNF and 20 pg/ml IL-1 β after 24 hours, but macrophages released high levels of TNF and IL-1 β in response to bacterial infection (Fig. 2E).

We also evaluated the differential expression of genes associated with apoptosis, including *TRAIL*, *XAF1* and *AIM2* genes by qRT-PCR. We found that these genes were upregulated following 8 and 18 hours of stimulation with *O. tsutsugamushi* (Fig. 2D). Interestingly, *O. tsutsugamushi* induced apoptosis in 13% and 18% of macrophages incubated with pathogens for 1 and 2 days, respectively, whereas only 1% of unstimulated macrophages and 3% of macrophages incubated with HK organisms were apoptotic (Fig. 3).

3.4. Macrophage response to heat-killed *O. tsutsugamushi*

To address the dependence of the macrophage response to *O. tsutsugamushi* on bacterial viability, we stimulated macrophages with heat-killed bacteria. In contrast to live *O. tsutsugamushi*, HK bacteria were unable to alter the type I IFN pathway; the expression of genes encoding IFN- β , OAS1 and MX1 was similar in macrophages stimulated with HK

1 bacteria compared to unstimulated macrophages (Fig. 4A), and HK bacteria did not induce
2 IFN- β release by macrophages (Fig. 4B). While genes associated with chemotaxis, such as
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4 *CXCL10* and *CXCL11*, were not modulated in response to HK *O. tsutsugamushi*, genes
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6 encoding TNF, IL-6, IL-12p40 and IL-15 were upregulated (Fig. 4A). This response was
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8 however reduced compared to that induced by live organisms. Finally, the expression of IL-
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10 1 β and IL-23p19 genes was similar in macrophages stimulated with live and HK organisms
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12 (compare Fig. 4A and Fig. 2C). Additionally, HK *O. tsutsugamushi* induced a lesser release
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14 of TNF than live organisms and were unable to induce the release of IL-1 β by macrophages
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16 (Fig. 4B). These results suggest that the type I IFN and, to a lesser degree, the response of
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18 macrophages to *O. tsutsugamushi* require live organisms.
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26 3.5. Effect of IFN- γ on the intracellular fate of *O. tsutsugamushi*

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28 As *O. tsutsugamushi* polarized macrophages toward an M1 phenotype, we evaluated whether
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30 the M1 polarization of macrophages by IFN- γ affected the intracellular fate of *O.*
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32 *tsutsugamushi*. On day 1, the number of copies of bacterial DNA in IFN- γ -treated
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34 macrophages decreased compared to day 0. However, the number of copies of bacterial DNA
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36 moderately increased on day 2 and day 3 to reach the level observed at day 0, and *O.*
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39 *tsutsugamushi* replicated within macrophages on days 4 and 5 (Fig. 5A). We expressed these
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41 results as the ratio of the number of copies of bacterial DNA in IFN- γ -treated macrophages to
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43 that of untreated macrophages. It was apparent that the treatment of macrophages with IFN- γ
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45 prior to infection did not alter the uptake of bacteria. In contrast, the replication of *O.*
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49 *tsutsugamushi* was significantly ($p < 0.05$) decreased in IFN- γ -treated macrophages compared
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51 to untreated macrophages throughout the infection (Fig. 5B), but it is important to note that
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53 the pre-treatment of macrophages with IFN- γ did not induce the complete elimination of *O.*
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1 *tsutsugamushi*. Together, these results suggest that the intracellular survival of *O.*
2 *tsutsugamushi* is associated with the polarization status of macrophages.
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7 **4. Discussion**

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9 We showed in this study that *O. tsutsugamushi* is able to replicate within human
10 macrophages. These results are consistent with previous data demonstrating that *O.*
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12 *tsutsugamushi* is present in tissue macrophages from autopsied patients with scrub typhus [8,
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14 25]. The survival of *O. tsutsugamushi* within macrophages may contribute to infection in
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16 organs such as the liver, spleen and lymph nodes in patients with scrub typhus.
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21 The mechanisms of *O. tsutsugamushi* internalization and survival in macrophages are
22 unknown; however, they appear to differ in part from those in monocytes. For example, the
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24 uptake of *O. tsutsugamushi* by macrophages is lower than that observed in monocytes, but *O.*
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26 *tsutsugamushi* replicates to a higher level in macrophages than in monocytes [13]. The
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28 absence of lipopolysaccharide and peptidoglycan in the *O. tsutsugamushi* cell wall suggests
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30 that Toll-like receptors do not play a role in the uptake of *O. tsutsugamushi* [9]. Nevertheless,
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32 heparin sulfate proteoglycans [9] and nucleotide-binding oligomerization domain-containing
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34 protein (NOD) 1 [26] may be involved in the recognition of extracellular and cytosolic *O.*
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36 *tsutsugamushi*, respectively, by endothelial cells. It is supposed that the bacterial 56-kDa
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38 type-specific antigen and surface antigen C (ScaC) are involved in cell invasion [9]. In non-
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40 professional phagocytes [27] and polymorphonuclear leukocytes [28], *O. tsutsugamushi* is
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42 localized to the cytosol. It is likely, therefore, that *O. tsutsugamushi* virulence is associated
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44 with escape from the phagosome in macrophages, potentially under the control of the
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46 bacterial genes encoding TlyC and a putative phospholipase D [9].
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56 Microarray analysis of infected macrophages revealed that *O. tsutsugamushi* induced
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58 sustained changes in the transcriptional program of these cells, including the upregulation of
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1 type I IFN genes and several ISGs. This long-lasting transcriptional effect was confirmed by
2 qRT-PCR and at the protein level as demonstrated by the release of IFN- β . The type I IFN
3 response may be related to the cytosolic localization of *O. tsutsugamushi*. It is known that
4 viruses [29] and cytosolic bacteria [30, 31] activate the type I IFN pathway. HK bacteria,
5 which are degraded in phagolysosomes, were unable to modulate the type I IFN response and
6 induce IFN- β release by macrophages as was previously reported for monocytes [13].
7 Although the role of the type I IFN pathway in host defense is still ambiguous [32], it is
8 known that type I IFNs activate immune cells (cytotoxic T cells and NK cells) that are critical
9 for the clearance of rickettsial infections [33, 34], but these cytokines seem to be detrimental
10 to the host when infected with *Listeria monocytogenes* [35] or *Tropheryma whipplei* [30].
11 Recently, it has been proposed that type I IFNs serve as probacterial function through the
12 suppression of IL-1 produced by myeloid cells [36].

13 The microarray analysis also showed that *O. tsutsugamushi* induced the upregulation
14 of inflammatory cytokines and chemokines. It has been demonstrated that mice susceptible to
15 *O. tsutsugamushi* produce greater amounts of inflammatory cytokines and chemokines
16 compared to resistant mice [18]. The ability of *O. tsutsugamushi* to induce the release of IL-
17 1 β suggests that *O. tsutsugamushi* activates the inflammasome [37], and that this response
18 requires bacterial viability, in contrast to the other components of the inflammatory response.
19 To define the inflammatory response of macrophages to *O. tsutsugamushi* within the context
20 of M1/M2 polarization, we compared the transcriptional program of *O. tsutsugamushi*-
21 stimulated macrophages with genes characteristic of M1 and M2 polarization [21-23]. We
22 found that *O. tsutsugamushi* induced essentially an M1-type program in macrophages, which
23 differ slightly from that elicited by *O. tsutsugamushi* in monocytes [13]. The upregulation of
24 genes encoding CCR7, IL-2 receptor α chain and IL-23p19 was more pronounced in
25 macrophages than in monocytes, whereas the expression of IL-6 was strikingly higher in

monocytes than in macrophages. The transcriptional program of infected macrophages resulted in a biological response, as shown by the release of TNF and IL-1 β by macrophages stimulated with *O. tsutsugamushi*. The high levels of TNF and IL-1 β release suggested a poor role for immunoregulatory cytokines such as IL-10. It has been previously shown that IL-10 may play a role in *O. tsutsugamushi* infection. Indeed, the preincubation of J774 macrophages with *O. tsutsugamushi* inhibited LPS-induced production of TNF through IL-10 [38]. We found that the expression of the gene encoding IL-10 was not modulated in response to *O. tsutsugamushi* as determined by microarray. We hypothesize that the imbalance between inflammatory and immunoregulatory cytokines is subtle. First, J774 macrophages incubated with *O. tsutsugamushi* for 24 hours released high levels of both TNF and IL-10 [38]. Second, in patients with scrub typhus, higher concentrations of IFN- γ and IL-10 were found during the acute phase of the disease compared with the convalescent phase, but the concentrations of TNF and IL-1 β were similar in acute and convalescent phases of the disease [17].

M1 polarization of macrophages, which is typically induced by IFN- γ in the presence of bacteria, causes these cells to become microbicidal toward intracellular microorganisms [19, 39]. We therefore evaluated whether the pre-treatment of macrophages with IFN- γ affected *O. tsutsugamushi* survival. We found that treatment with IFN- γ led to reduced *O. tsutsugamushi* replication without eradicating the bacteria. Thus, despite the detrimental effect of M1 polarization on *O. tsutsugamushi* replication, the bacteria nevertheless induced this M1 polarization in macrophages. However, it is likely that other elements of the host response to *O. tsutsugamushi* contribute to bacterial replication. These factors include type I IFNs, which have been shown to be critical for the survival of *Mycobacterium tuberculosis* [36]. In addition, apoptosis may favor the replication of *O. tsutsugamushi*. We observed that *O. tsutsugamushi* infection was associated with the up-regulation of apoptosis-related genes and with the induction of apoptosis as seen in the TUNEL assay. *O. tsutsugamushi* induced

apoptosis in macrophages to a greater degree than in monocytes, and the replication of *O. tsutsugamushi* was higher in macrophages than in monocytes [13], suggesting that bacterial replication and apoptosis are associated.

5. Conclusions

We demonstrated that human macrophages are targeted in vitro by *O. tsutsugamushi*, reinforcing the idea that tissue macrophages may be infected during scrub typhus. In macrophages, *O. tsutsugamushi* stimulated a type I IFN response, likely due to its cytoplasmic localization and its induction of an M1-type response. This exacerbated inflammatory response may be related to the inflammatory complications observed in scrub typhus.

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Legends of Figures

Figure 1. *O. tsutsugamushi* infection of macrophages and microarray analysis

A, Macrophages were incubated with *O. tsutsugamushi* (10 bacteria per cell) for 2 hours, permeabilized and incubated with Alexa Fluor 488-phalloidin and with antibodies specific for *O. tsutsugamushi*. Cells were examined by confocal microscopy, and a representative micrograph is shown with filamentous actin in green and bacteria in red. **B**, Macrophages that were incubated with *O. tsutsugamushi* for 2 hours were washed to remove free organisms and were cultured for an additional 5 days. The number of copies of bacterial DNA was determined by qPCR. The results are the mean \pm SD of three different experiments. **C**, Genes modulated in macrophages that were stimulated with *O. tsutsugamushi* for 8 hours are represented as a heatmap with probes in rows and samples in columns. Dendograms show the results of the hierarchical clustering of the samples (top) and genes (left). Gene expression levels are color-coded from blue to red. **D**, Genes modulated in response to *O. tsutsugamushi* were analyzed using DAVID functional analysis tools. The results are displayed as a pie graph representing the genes that were specifically modulated.

Figure 2. Effect of *O. tsutsugamushi* on the transcriptional response of macrophages

A-D, Macrophages were stimulated with *O. tsutsugamushi* for 8 and 18 hours. RNA was extracted and qRT-PCR was performed on genes found to be regulated in the microarray analysis, including genes associated with type I IFN (A) and chemotaxis (B), genes encoding cytokines (C) and genes associated with apoptosis and microbicidal responses (D). Results are expressed as the relative expression of each gene (stimulated vs. unstimulated conditions) and represent the mean \pm SD of 3 independent experiments performed in triplicate. **E**, Macrophages were stimulated with *O. tsutsugamushi* for different time periods. Culture

supernatants were analyzed for the presence of IFN- β , TNF and IL-1 β using specific immunoassays. The results are expressed in pg/ml and are presented as the mean \pm SD of three experiments performed in duplicate.

Figure 3. Effect of *O. tsutsugamushi* on macrophage apoptosis

Macrophages were stimulated with *O. tsutsugamushi* for 2 days, and cell apoptosis was determined by TUNEL assay. **A**, unstimulated macrophages. **B**, *O. tsutsugamushi*-stimulated macrophages.

Figure 4. Macrophage responses to HK *O. tsutsugamushi*

A, Macrophages were stimulated with HK *O. tsutsugamushi* for 8 hours. RNA was extracted and qRT-PCR was performed. The results are expressed as the relative expression of each gene (stimulated vs. unstimulated conditions) and represent the mean \pm SD of 3 independent experiments performed in triplicate. **B**, Macrophages were stimulated with *O. tsutsugamushi* for different time periods. Culture supernatants were analyzed for the presence of cytokines using immunoassays. The results are expressed in pg/ml and are presented as the mean \pm SD of three experiments performed in duplicate.

Figure 5. Effect of IFN- γ on the intracellular fate of *O. tsutsugamushi*

A, Macrophages were pre-treated with 50 ng/ml IFN- γ for 24 hours and were infected with *O. tsutsugamushi* (bacterium-to-cell ratio of 10:1) for 2 hours. After washing, macrophages were cultured for 5 days in the presence of IFN- γ . The number of copies of bacterial DNA was determined by qPCR. The results are the mean \pm SD of three different experiments. **B**, Macrophages pre-treated with IFN- γ and control (untreated) macrophages were infected with *O. tsutsugamushi* as described above. The results are expressed as the ratio of the number of

copies of bacterial DNA in IFN- γ -treated macrophages compared to control macrophages and
the mean \pm SD of three different experiments.

Reviewer #1

1. p5, 2.1, line 6, the authors mentioned that they used CD14 and CD68 to differentiate monocytes from macrophages and there was more than 90% of the cells used were macrophages. It was not clear how this was determined, by flow cytometry? If so, can the author show the data after sorting as this is crucial for the experiment below?

The method (flow cytometry) to determine the percentage of macrophages is indicated in the new manuscript (page 5, lines 11-12). A representative experiment is also shown as supplementary data including how macrophages were collected (Figure S1).

2. p.5, line 8, IFN-gamma was maintained during the course of the experiment. Is there evidence to show that the recombinant IFN-gamma was stable for 24 hour at 1000U/ml under the culturing condition?

We previously showed that 1000 UI/ml IFN-gamma was effective to activate macrophages (ref 19). A concentration of 20 ng/ml IFN-gamma was recently used to activate human macrophages (Ouedraogo et al. J Proteomics 2012, in press): this concentration corresponded to 400 UI/ml IFN-gamma. To ensure that IFN-gamma was efficient, macrophages were pretreated with 1000 UI/ml IFN-gamma for 24 hours, infected for 2 hours, and incubated with 1000 UI/ml IFN-gamma every day. As the relationship between IFN-gamma activity and IFN-gamma doses is not clear, we replace 1000 UI/ml by 50 ng/ml IFN-gamma. These points are indicated in 2.1. (page 5, lines 13-14) and the legend of figure 5.

3. p.6. A known concentration of bacterial DNA. How the concentration of bacteria (Orientia) DNA was determined? The qPCR data, including the standard curve, should be provided as a supplement figure.

In a preliminary experiment, *Orientia* was propagated in L929 cells, and bacteria were isolated after cell lysis. The bacterial preparation was divided into two parts: the first one was used to isolate bacterial DNA using a QIAamp Tissue Kit as described in the manuscript and the second part was used to determine the bacterial concentration in this preparation. For that purpose, serial dilutions of the bacterial preparation were deposited on glass coverslips (under a known volume), and the concentration of bacteria was determined by indirect immunofluorescence with pooled serum from patients with scrub typhus and secondary FITC-antibodies. A very simple calculation (one specific DNA sequence per bacterium) allows the determination of bacterial DNA concentration per sample.

The qPCR data including the standard curve are now presented in 2.2. (page 6, lines 9-17) and as a supplemental figure (Figure S2).

4. The bacterium-to-cell ratio in 2.2 and 2.3 was different (10:1 to do infection and 20:1 to prepare samples for microarrays). Can the authors provide a rationale?

The bacterium-to-cell ratio in 2.3 was wrong. It was 10:1 in both 2.2 and 2.3., and corrections are included in the new manuscript (2.3. page 6, line 21).

5. p6, line 13. RNA was amplified. How RNA was amplified?

The sentence has been modified in 2.3. (page 7, line 1).

6. p7. FC in the expression of genes relative to the expression of β -actin was computed. The basic assumption by the authors here was: The expression of β -actin gene is not altered upon infection with *Orientia* over the time course studied. Is there any evidence to support this assumption? It has been observed that the actins are involved in the cytoskeletal changes in rickettsia infected cells. It is possible that the expression level of β -actin might be changed. A better normalization standard is 18S RNA gene.

We agree that the expression of b-actin may be altered by *Orientia* infection. We studied the expression of b-actin gene by unstimulated and *Orientia*-stimulated macrophages using microarray. We found no difference. We also tested the gene encoding GAPDH, another housekeeper gene. Its expression was similar in unstimulated and *Orientia*-stimulated macrophages, demonstrating that the genes encoding b-actin and GAPDH are relevant to quantify qRT-PCR data. This precision has been added in 2.4. (page 8, lines 3-5).

7. p.8, Figure 1 D. The criteria for the selection of modulated genes should be clearly stated. There were four samples per experiment condition (p6). What is the consistency in expression levels among the results from four quadruplet samples? Were the results in figure 1C derived from the average of four sets of data or a representative of one sample?

We did not use four quadruplet samples, but four samples per experimental condition (unstimulated macrophages and *Orientia*-stimulated macrophages). The reproducibility of these samples was shown in Figure 1C, and the inter-array values were normalized using the Rosetta intensity error model for single color microarrays. These precisions have been added in 2.3. (page 7, lines 7-8).

The results in Figure 1D were derived from the average of the four sets of data. This information has been added in 2.3 (page 7, lines 11-13).

8. The regulation of M1 genes and M2 genes (Figure 3) should be presented as a table listing all those regulated genes.

The M1 genes and the M2 genes are presented in the new manuscript as Table 2 and Table 3, respectively.

9. In the results session, 3.3 on page 10 line #14, it should be Figure 2D rather than Figure 3D. Furthermore, the percentage of apoptotic cells were 12% and 17% in *Orientia* infected macrophages but only 2% in HK *Orientia*. What is the background level of apoptosis in macrophages without any stimulation? Or was the 12% already corrected for the background? Since the data is available, the reviewer would suggest showing the image of apoptotic cells as demonstrated by TUNEL assay.

Fig3D has been replaced by Fig2D.

The background level of apoptosis in unstimulated macrophages was about 1%. The percentage of apoptotic cells is presented in the new section 3.3 (page 11, lines 17-19). The images of unstimulated and *Orientia*-stimulated macrophages are presented as a new Figure (Figure 3).

10. For Figure 2E and 4B, it appeared that only the results of the IFN- β , TNF and IL-1 β from supernatant of *Orientia* infected and HK *Orientia* infected macrophage are shown. What about the unstimulated samples since the case was made that a significant increase was observed, it is important that the readers can see the background level (if any). In addition,

IFN- β is the only one that had two time points of 24 and 48 h while the other two had 8 and 24 h time points. Can the authors provide a justification for the time difference?

Unstimulated macrophages did not release IFN- β (less than 8 pg/ml). This information was in the new 3.3. (page 10, lines 19-20). They released low levels of TNF or IL-1 β , and this information is given in the new paragraph 3.3. (page 11, lines 11-12). In addition, the detection limits and intra and interassay coefficients of variation of ELISA kits is given in the new paragraph 2.6 (page 8, lines 22-24).

In response to numerous pathogens, the inflammatory response occurs some hours after bacterial stimulation (justifying 8 and 24 hours) and decreased thereafter. The IFN response is delayed as compared to TNF response, and its maximum is comprised between 24 and 48 hours. The release of TNF and IL-1 β of macrophages stimulated with *Orientia* was studied at 8 and 24 hours, as previously described for monocytes stimulated with *Orientia* (see ref 13). As the activation of IFN- β gene was sustained (upregulation at 8 and 24 hours), we tested the release of IFN- β not only at 24 hours (see ref 13) but also at 48 hours. We found no difference between 24 and 48 hours.

11. In Figure 5B, the "100% IFN+/IFN- ratio was obtained after pretreating host cells with IFN (or not) for the same period of time, incubated with Orientia (a given number of what ratio?) followed by washing to remove unassociated Orientia then extract DNA from the infected cells to determine the # of Orientia organisms? For Figure 5B, should the Y axis be specified $\times 10^4$ or something similar as shown in Figure 1B? How the concentration of 1000U/ml of IFN- γ was determined? Is it possible to carry out the experiment at a higher concentration to totally eradicate Orientia?

The bacterium-to-cell ratio (10:1) is now presented in the legend of figure 5.

The precise value of bacterial DNA copies was lacking on the Y axis of figure 5A. This value is specified in the new figure 5A.

The concentration of IFN- γ was highly effective. The concentration of 1000 UI/ml was higher than that used by Dellacasagrande et al. (J. Immunol. 2002,169:6309-15) and Ouedraogo et al. (J. Proteomics, 2012, in press). We also added IFN- γ to macrophages every day whereas Benoit et al. (PLoS Pathog. 2008, 4:e1000066) added IFN- γ every three days. The bacterial replication observed from day 4 to day 5 was not due to a lack of IFN- γ because IFN- γ was added to infected macrophages every day. This information has been added in 2.1. (page 5, line 14).

12. Many experiment details were referred to previous study (Ref 13). However, important information should still be included in this MS.

Experiment details are included in the new manuscript. See 2.1. (page 4, lines 21-24 and page 5, line 1); 2.2. (page 6, lines 9-17); 2.3. (page 6, lines 23-25; page 7, lines 3, 7-8 and 11-13); 2.4. (page 8, lines 6-8); 2.5. (page 8, lines 13-14); 2.6. (page 8, lines 22-24). We also added two references (ref 22 and 23) to describe the selection of M1/M2 genes.

Discussion:

1. At the end of discussion, a comment was made about bacterial replication within host cells and apoptosis of host cells are associated. From day 4 to day 5, the increase in DNA copies was about 3 fold. Does this indicate the adaptation of Orientia in cultured macrophages? Can the authors speculated any survival advantage for this to occur in the macrophage at faster

rate (both apoptosis and replication) or does it mean macrophages may not be a "natural" host cells for Orientia infection although in vitro it can be used as a host cell?

The number of bacterial DNA copies increased 3 fold from day 4 to day 5 in macrophages treated or not with IFN-gamma, suggesting the adaptation of Orientia to macrophages. This rate of replication, after a transient eclipse, was similar to that found in L929 cells but largely decreased compared to that observed in monocytes (see ref 13). On the other hand, Orientia induced a higher level of apoptosis in macrophages than in monocytes (compare our results with ref 13). The decreased bacterial replication and the increased apoptosis in macrophages compared with monocytes suggest that both events are associated.

The hypotheses of the reviewer are interesting. As macrophages seem to be a natural host for Orientia (see reference 8 for human infection and reference 10 for murine infection), we believed rather to an adaptation of Orientia to macrophages.

However, the underlying mechanisms and the biological signification of the interaction between macrophages and Orientia are unknown. As they are not under the scope of our paper, we prefer to avoid speculations.

2. IL-10 secretion was observed in murine macrophages(J774, Kim MJ et al Microbial Pathogenesis 2005) and in acute patient samples (Ref 17, 2009). Orientia inhibits TNF- α by inducing IL-10 secretion. It has been shown that TNF- α was associated with the scrub typhus diseases severity.

Why IL-10 was not selected for this study. Is it because the level of IL-10 was not changed in this study? At least the potential effect of IL-10 should be discussed.

We found that the gene encoding TNF and genes encoding inflammatory cytokines and chemokines were up-regulated in O. tsutsugamushi-stimulated macrophages. The release of TNF was also dramatically increased in infected macrophages, suggesting a poor role for immunoregulatory cytokines such as IL-10. In addition, the expression of IL10 gene was not modulated in response to O. tsutsugamushi as determined by microarray.

The potential role of IL-10 in O. tsutsugamushi infection has been added in the new Discussion (4. page 15, lines 3-13) with a new reference (ref 38).

e-component

[Click here to download e-component: FigS1PDF.pdf](#)

e-component

[Click here to download e-component: FigS2PDF.pdf](#)

Figure

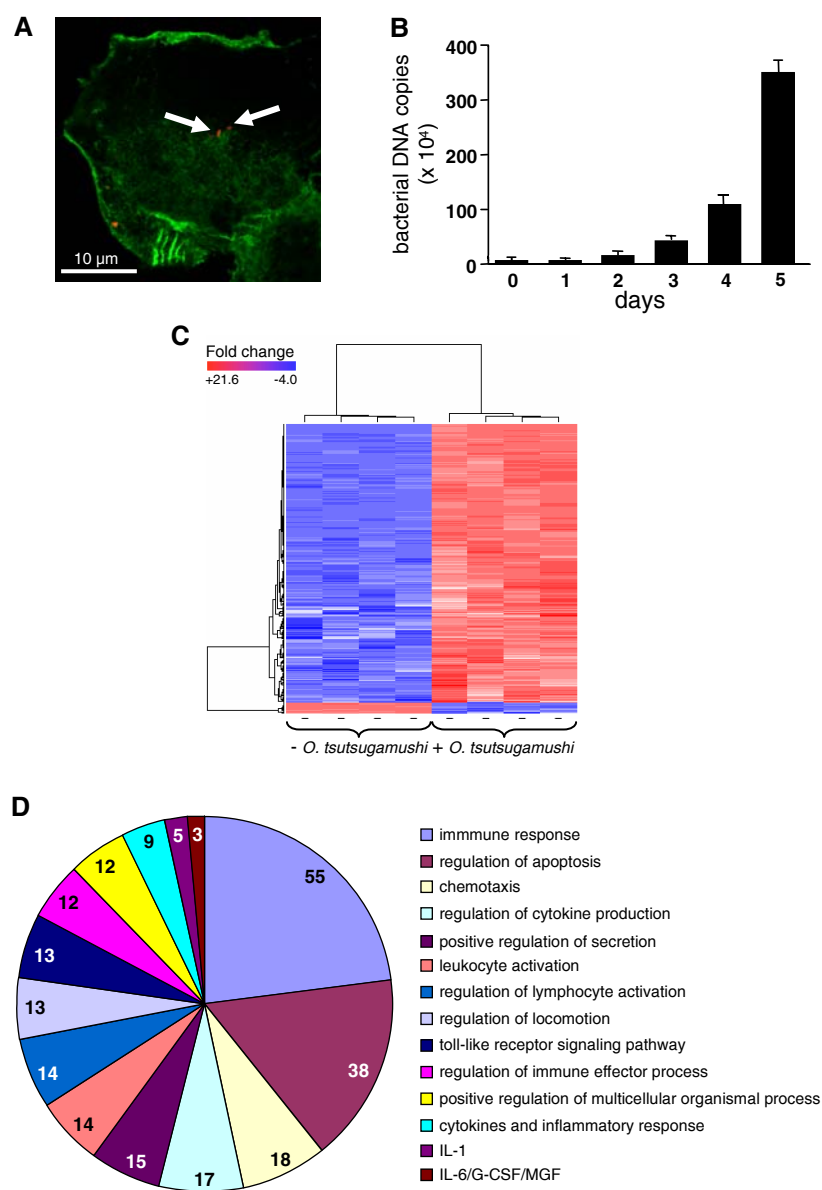


Figure 1

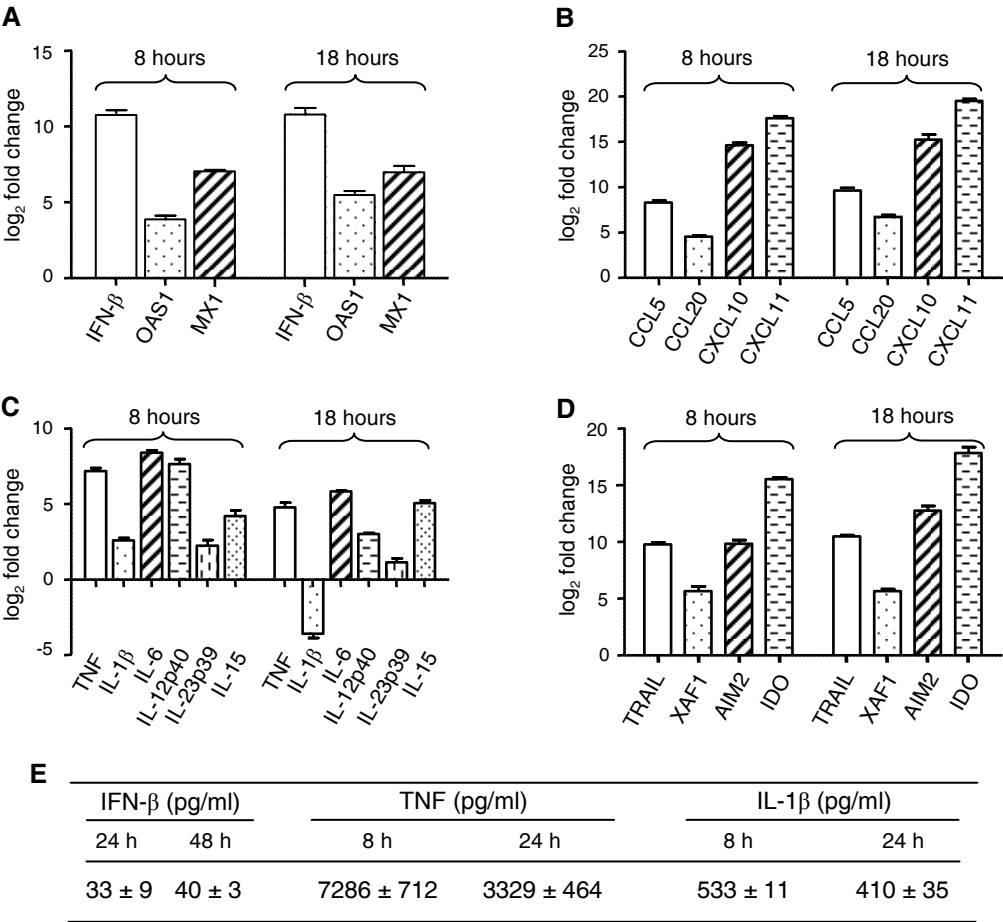
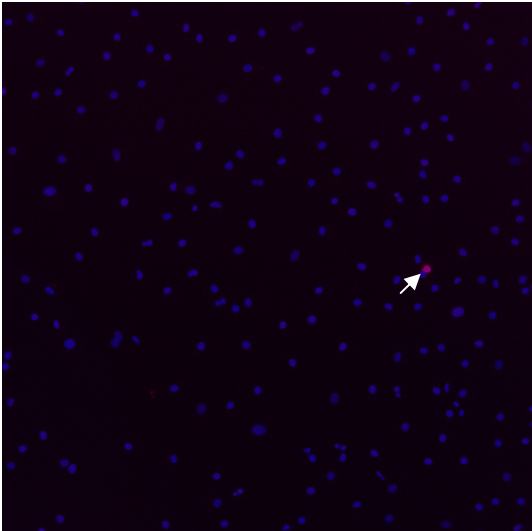


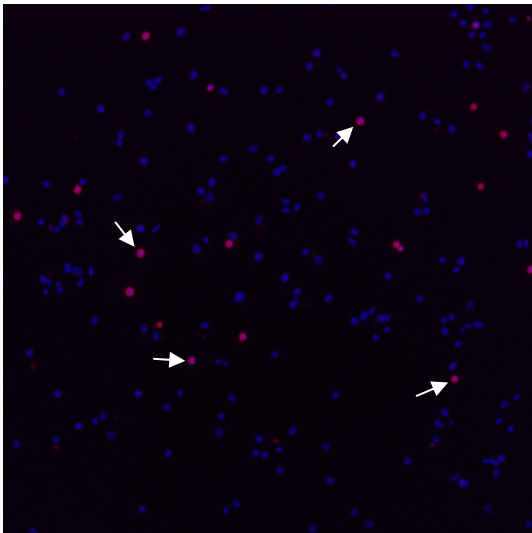
Figure 2

Figure

A



B



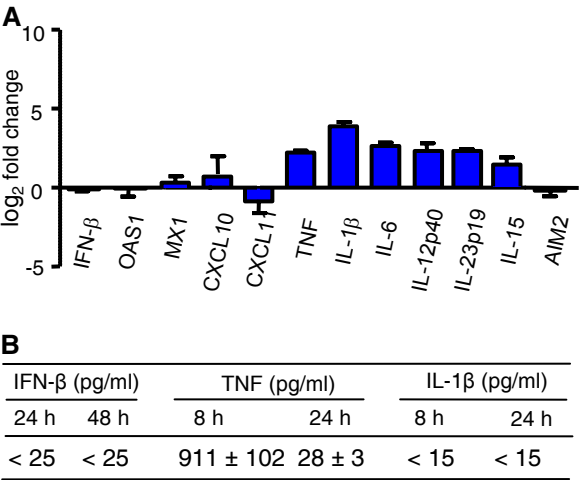


Figure 4

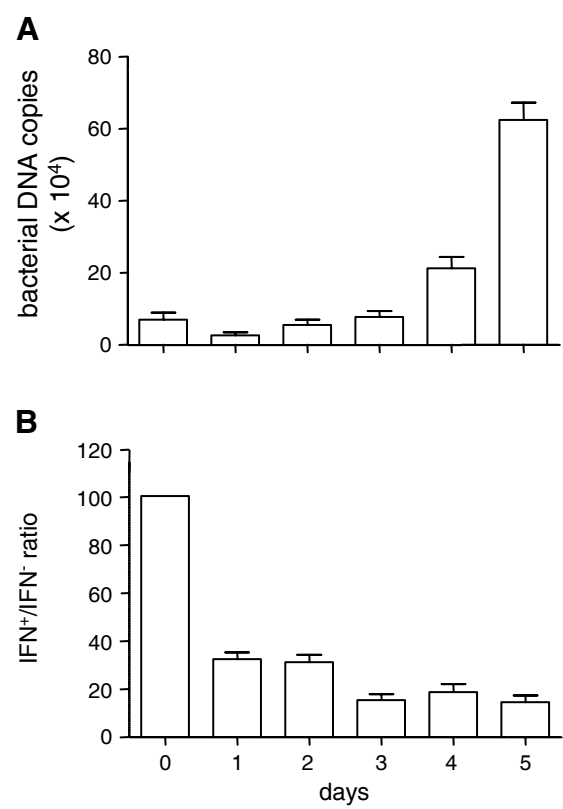


Figure 5

Table 1. Nucleotide sequences of oligonucleotide primers

Symbol	5'-3' forward primers	5'-3' reverse primers
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
AIM2	GGTTTGTGGTAGTCCAGAAGGTA	GTGCTATGAACTCCAGATGTCAG
CCL5	CTGCTGCTTTGCCTACATTGC	G TTCAGGTTCAAGGACTCTCCATC
CCL20	GACATCAATGCTATCATCTTTTAC	GCTATGTCCAATTCCATTCCA
CXCL10	AAGCAGTTAGCAAGGAAAGGTC	TTGAAGCAGGGTCAGAACATC
CXCL11	TATAGCCTTGGCTGTGATATTGTG	CTGCCACTTTCACTGCTTTTACC
IDO	TGCTGGTGGAGGACATGCTG	TGAAAGGACAACTCACGGACTGA
IFN β	GACGCCGCATTGACCATCTA	CCTTAGGATTTCCACTCTGACT
IL-1 β	GCTGAGGAAGATGCTGGTTC	TCCATATCCTGTCCCTGGAG
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT
IL-12p40	AGGGGACAACAAGGAGTATGAGT	AGGGAGAAGTAGGAATGTGGAGT
IL-15	GTTAGCAGATAGCCAGCCCATAC	TACTCAAAGCCACGGTAAATCC
IL-23p19	AGGAGAAGAGGGAGATGAAGAGAC	GCTATCAGGGAGCAGAGAAGG
MX1	GCCACCACAGAGGCTCTCAG	CTCAGCTGGTCCTGGATCTCCT
OAS1	TCCGCCTAGTCAAGCACTGGTA	CCTGGGCTGTGTTGAAATGTGT
TNF	CCCGACTATCTCGACTTTGC	AGGTTGAGGGTGTCTGAAGGA
TRAIL	GGCTAACTGACCTGGAAAGAAA	TTGGTTGTGGCTGCTCTACT
XAF1	GAGCTCCACGAGTCCTACTGT	CTCTGAGTCTGGACAACATTTACC

Table 2. Modulation by *O. tsutsugamushi* of M1-related genes

Gene Symbol	NCBI Gene ID	ENSEMBL Gene ID	PROBE_ID	FC
CCR7	1236	ENSG00000126353	A_23_P343398	8.31
IL2RA	3559	ENSG00000134460	A_23_P127288	3.12
IL7R	3575	ENSG00000168685	A_23_P404494	1.76
IL15RA	3601	ENSG00000134470	A_23_P138680	2.70
CCL2	6347	ENSG00000108691	A_23_P89431	1.31
CCL3	6348	ENSG00000006075	A_23_P373017	4.54
CCL4	6351	ENSG00000129277	A_23_P207564	2.52
CCL5	6352	ENSG00000161570	A_23_P152838	1.94
CCL15	6359	ENSG00000161574	A_24_P301501	1.22
CCL19	6363	ENSG00000172724	A_23_P123853	1.86
CCL20	6364	ENSG00000115009	A_23_P17065	2.01
CXCL9	4283	ENSG00000138755	A_23_P18452	1.46
CXCL10	3627	ENSG00000169245	A_24_P303091	3.49
CXCL11	6373	ENSG00000169248	A_24_P20607	11.09
CXCL16	58191	ENSG00000161921	A_23_P38505	1.17
IL8	3576	ENSG00000169429	A_32_P87013	3.18
TYMP	1890	ENSG00000025708	A_23_P91802	1.13
IL1A	3552	ENSG00000115008	A_23_P72096	4.82
IL1B	3553	ENSG00000125538	A_23_P79518	5.92
IL6	3569	ENSG00000136244	A_23_P71037	3.47
IL12A	3592	ENSG00000168811	A_23_P91943	1.31
IL12B	3593	ENSG00000113302	A_23_P7560	17.81
IL23A	51561	ENSG00000110944	A_23_P76078	5.12
TNF	7124	ENSG00000232810	A_24_P50759	3.03
TNFSF10	8743	ENSG00000121858	A_23_P121253	5.23
BCL2A1	597	ENSG00000140379	A_23_P152002	1.32
FAS	355	ENSG00000026103	A_23_P63896	2.05
BIRC3	330	ENSG00000023445	A_23_P98350	1.43
GADD45G	10912	ENSG00000130222	A_24_P120934	1.25
XAF1	54739	ENSG00000132530	A_23_P4283	2.64

Macrophages were stimulated with *O. tsutsugamushi* for 8 hours, and microarrays were performed. M1-related genes (n = 30) were manually selected from the literature [21-23]. The reference IDs of genes at NCBI and ENSEMBL are provided, as well as the probe ID on microarray. The ratio of gene expression of stimulated monocytes to control monocytes (FC) is shown.

Table 3. Modulation by *O. tsutsugamushi* of M2-related genes

Gene symbol	NCBI Gene ID	ENSEMBL Gene ID	PROBE_ID	FC
CD36	948	ENSG00000135218	A_23_P111583	0.72
CD163	9332	ENSG00000177575	A_23_P33723	0.75
CD209	30835	ENSG00000090659	A_24_P186539	0.90
CXCR4	7852	ENSG00000121966	A_23_P102000	1.42
CD302	9936	ENSG00000241399	A_24_P396702	0.79
CLEC7A	64581	ENSG00000172243	A_23_P2640	0.73
P2RY14	9934	ENSG00000174944	A_24_P165864	0.47
HRH1	3269	ENSG00000196639	A_23_P212179	0.81
MSR1	4481	ENSG00000038945	A_23_P216176	1.13
MS4A4A	51338	ENSG00000110079	A_23_P75769	0.67
MS4A6A	64231	ENSG00000110077	A_23_P203376	0.52
LPAR6	10161	ENSG00000139679	A_23_P2705	0.68
TGFBR2	7048	ENSG00000163513	A_23_P211957	0.47
CCL1	6346	ENSG00000108702	A_23_P49759	2.79
CCL13	6357	ENSG00000181374	A_23_P26965	0.60
CCL17	6361	ENSG00000102970	A_23_P26325	0.91
CCL18	6362	ENSG00000006074	A_23_P55270	1.11
CCL22	6367	ENSG00000102962	A_24_P313418	0.95
CCL23	6368	ENSG00000167236	A_24_P133905	1.03
CCL24	6369	ENSG00000106178	A_23_P215491	1.05
IL1RN	3557	ENSG00000136689	A_32_P38125	3.05
IL10	3586	ENSG00000136634	A_23_P126735	1.12

Macrophages were stimulated with *O. tsutsugamushi* for 8 hours, and microarrays were performed. M2-related genes (n = 22) were manually selected from the literature [21-23]. The reference IDs of genes at NCBI and ENSEMBL are provided, as well as the probe ID on microarray. The ratio of gene expression of stimulated monocytes to control monocytes (FC) is shown.